EXHIBIT A

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FIP: a novel approach to vaccination

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Summary Feline infectious peritonitis (FIP) is a fatal disease of cats. Early attempts at vaccination have been unsuccessful, some even serving to exacerbate the disease through antibody-dependent enhancement. Replication-incompetent feline foamy virus (FFV) transducing vectors are being developed as potential vaccine agents, into which immunogenic fragments of feline coronavirus (FCoV) proteins will be inserted. To use a recombinant viral vector to express FCoV proteins, the agent chosen should be apathogenic and replication incompetent within the host following gene delivery. Spumaviruses confer several advantages over the more traditionally explored retroviral vectors. Stable helper cell line clones have been established by transfection of CRFK cells with FFV *tas* and assessed using β-galactosidase assays, PCR, immunofluorescence and western blotting. The generation of infectious virions using these cell lines has been investigated using tas-deleted FFV vectors containing the enhanced green fluorescent protein (eGFP) cassette.

Introduction

Feline infectious peritonitis (FIP) is a fatal disease of cats caused by feline coronavirus (FCoV) infection. There are two serotypes (Fiscus and Teramoto, 1987; Hohdatsu et al., 1991), type I being more prevalent in field conditions (Hohdatsu et al., 1992), accounting for at least 70% of FIP cases. However, to date, most vaccine attempts have been based on serotype II. Development of vaccines has been largely unsuccessful, with traditional approaches sometimes serving to exacerbate disease through antibody dependent enhancement (ADE). The identification of FCoV epitopes responsible for neutralisation and enhancement is still under investigation. Attempts at vaccination have included the use of avirulent FCoV (Pedersen and Black, 1983), recombinant vaccina virus expressing the S protein (Vennema et al., 1990), non-feline coronaviruses (Barlough et al., 1984, 1985; Stoddart et al., 1988) and plasmids expressing M or N proteins alone, or with enhancement through vaccinia viruses or feline cytokines (Glansbeek et al., 2002). A commercial vaccine (Christianson et al., 1989) was launched in 1991 (Primucell-FIP™, Pfizer Animal Health), however, its safety remains controversial and it is not licensed for kittens less than 16 weeks old (McArdle et al., 1995; Scott et al., 1995).

Generation of mucosal immunity may be critical to prevent FCoV infection and FIP. A strong cellular immune response, through the induction of type I immunity, appears to be the key to virus control (reviewed by Olsen, 1993). Minimisation of the development of humoral antibodies may be valuable in avoiding ADE of infection and immune complex deposition. Expression of serotype 1 FCoV

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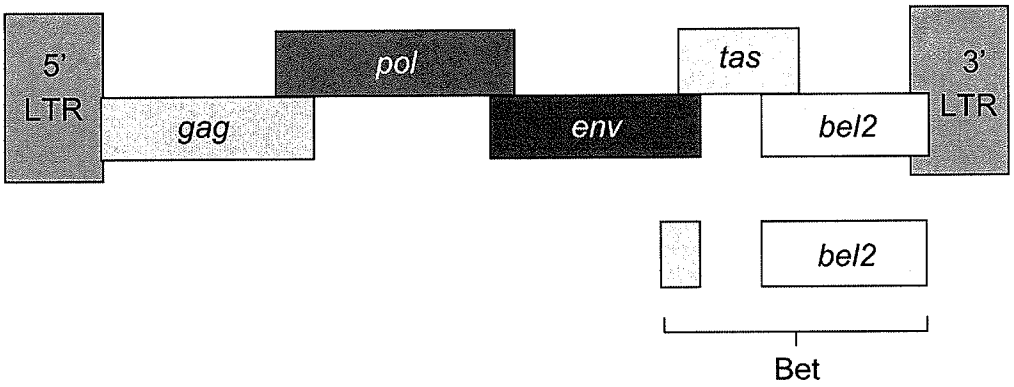

Figure 1 Diagrammatic representation of the feline foamy virus (FFV) genome. FFV has the typical retroviral organisation, consisting of a 5′ and 3′ LTR and the main structural genes *gag*, *pol* and *env*. It also contains two accessory open reading frames (*bel1/tas* and *bel2*), as compared to prototypic and simian foamy viruses, which have three. *tas* encodes for the viral transactivator Tas, which drives temporal expression from the internal promoter (IP) at the 3′ end of *env* and the 5′ LTR U3 promoter. Bet is produced as a splice protein from the 5′ region of *tas* and *bel2*, as indicated.

epitopes that induce a protective response using a virus vector from a different family may provide a solution and would reduce the risk of virus recombination. Retroviral vectors may act as suitable candidates as they are persistent, integrating a proviral copy of their genome into the host DNA, from which protein expression occurs whenever transcription of that region is initiated. Spumaviruses offer several potential advantages over the more traditionally explored retroviral vectors. They have a large genome size; a wide cellular host range; are relatively apathogenic and require the presence of the viral transactivator (Tas) for high level transcription from the viral long terminal repeat (LTR). The latter factor enables the development of replication-incompetence, as deletion of the transactivator prevents transcription of the structural genes. Supplying this protein *in trans*, via a helper cell line, allows the production of an infectious viral particle able to infect a susceptible cell and integrate. However, such an infection would be abortive, unable to produce infectious virions because of the lack of Tas in the target cell.

This paper describes the initial phases in the development of feline foamy virus vectors for investigation as candidate vaccines for FIP, using a Tas-dependent, replication incompetent system.

Materials and methods

Construction of the helper cell line

FFV strain F-17 has been sequenced (Helps and Harbour, 1997) and the genomic organisation determined (Fig. 1). *tas* was amplified from the F-17 genome by polymerase chain reaction (PCR), gel purified, restriction digested and ligated into a pHook-3 plasmid (Invitrogen, Paisley, UK) by standard techniques. The positive control plasmid, pHook-3/His/LacZ (Invitrogen), contained the *lac Z* gene under the CMV promoter.

Plasmids were grown in *Escherichia coli* DH5a and purified as endotoxin-free (Endofree Plasmid Maxi Kit, Qiagen, Crawley, UK). Confirmation of the integrity of the plasmids was achieved through PCR (pHook-3/tas) and restriction digests (pHook-3/lacZ), with the products visualised by agarose gel electrophoresis. The functionality of these plasmids was investigated through transfection assays and β-galactosidase staining. A cationic lipid-based transfection system (Lipofectamine, Invitrogen) was used to carry plasmid DNA into FKCU/LTR/zeo/β-gal reporter cells (a kind gift from Prof. A. Rethwilm, University of Wurzburg, Germany). These cells contain the *lacZ* gene under the control of the FFV LTR. Production of functional Tas by transfected cells would result in transcription of the *lacZ* gene. The β-galactosidase produced catalyses the hydrolysis of β-galactosides. The substrate 'X-gal' (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) produces a blue coloured product when cleaved by β-galactosidase, which can be visualised by light microscopy.

Transfection of CRFK cells and cell cloning

CRFK cells were transfected with pHook3/tas plasmids in an optimised assay, the positive cells being selected through antibiotic resistance and serially diluted to obtain single cell clones. In parallel,

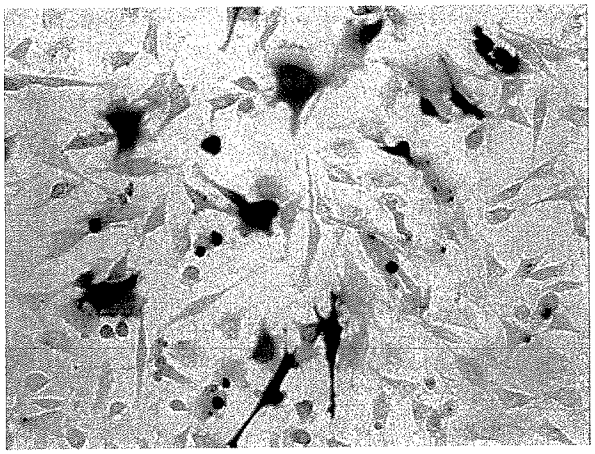

Figure 2 FKCU/LTR reporter cells, transfected with the pHook-3/tas plasmid and stained by the β-gal assay. Transfection efficiency approximated as 25–30%, based on the development of blue-coloured cells. (Magnification ×200).

CRFK cells were transfected with pHook3/βgal, dilution cloned, grown for 3 weeks and then stained to assess the effectiveness of the procedure.

Analysis of helper cell clones

Once single cell clones had been generated, they were analysed by immunofluorescence, PCR and through transfection assays. A second reporter plasmid was used for this purpose (pLTR1/(FFV)/β-gal/zeo, a kind gift from Prof. A. Rethwilm), which contained the *lacZ* gene under the control of the FFV U3-LTR promoter. If the helper cell line clone were to express functional Tas, the LTR promoter would be activated, which would allow expression from the *lacZ* gene. β-galactosidase production could then be assessed by the standard colourimetric assay. CRFK cells were transfected in parallel to act as a negative control.

Vector constructs, containing the eGFP gene in place of the viral transactivator, were used to investigate packaging ability and the generation of virions able to infect feline cells. Vectors were transfected into the helper cells using Lipofectamine (Invitrogen). Cells were passaged and monitored for the eGFP fluorescence.

Results

Optimisation of the transfection method resulted in transfection efficiencies averaging 30% (Fig. 2). Helper cell line clones (CRFK/tas) were developed over a 5-month period, with cell clones being grown to confluency, expanded, tested and frozen as stocks. Thirty-two CRFK/tas single cell clones were identified. Seven of these clones were found to express functional Tas, with efficiencies ranging from 25–60% (Fig. 3).

PCR of the functional helper cell line clones for the presence of the *tas* gene insert revealed all to be positive for a 600 b.p. band, consistent with the expected size of the *tas* gene. The homogeneity of expression of the helper cell lines has subsequently been assessed and further studies into Tas are underway.

Helper cell lines were transfected with the replication-incompetent, *tas*-deleted vectors, containing the eGFP gene cassette. Fluorescence was detected in 10–20% of cells using an inverted fluorescence microscope. The ability of these vectors to replicate in the helper cell lines and transfer to independent cell cultures is being investigated.

Discussion

Viral vectors have been developed for human gene therapy applications in recent years, though many current vehicles remain either inefficient or potentially unsafe. The unique biology of the foamy viruses lends themselves to development as such vectors, particularly their reported lack of pathogenicity. In humans, zoonotic infection with simian FV (SFV) has been reported and it is now thought that human FV (renamed prototypic (P)FV) was originally a zoonotic infection with SFVcpz (Herchenroder et al., 1994). SFV infection in man has been reported as apathogenic, in studies involving up to a 23-year monitoring period, and man appears to be a dead-end host (Heneine et al., 1998). Despite links to a number of diseases, including de Quervain thyroiditis (Werner and Gelderblom, 1979), Graves disease (Lagaye et al., 1992) and Ménière disease (Pyykkö et al., 1993), no studies have subsequently confirmed these associations (reviewed by Heneine et al., 2003). There have been reports of association of FFV with feline polyarthropathy (Pedersen et al., 1980) and a co-factor effect with FIV (Winkler et al., 1999; Zenger et al., 1993), but these reports have not been confirmed by other workers in the field and there is no convincing evidence of any direct pathology associated with infection. Studies have shown FFV to be relatively ubiquitous in the domestic and feral cat populations, with seroprevalences ranging from 5–100%, with over 50% for most populations (Bandecchi et al., 1992; Flower et al., 1985; Winkler et al., 1997, 1998, 1999). It has been concluded from phylogenetic analyses that simian foamy viruses have a long history of coexistence

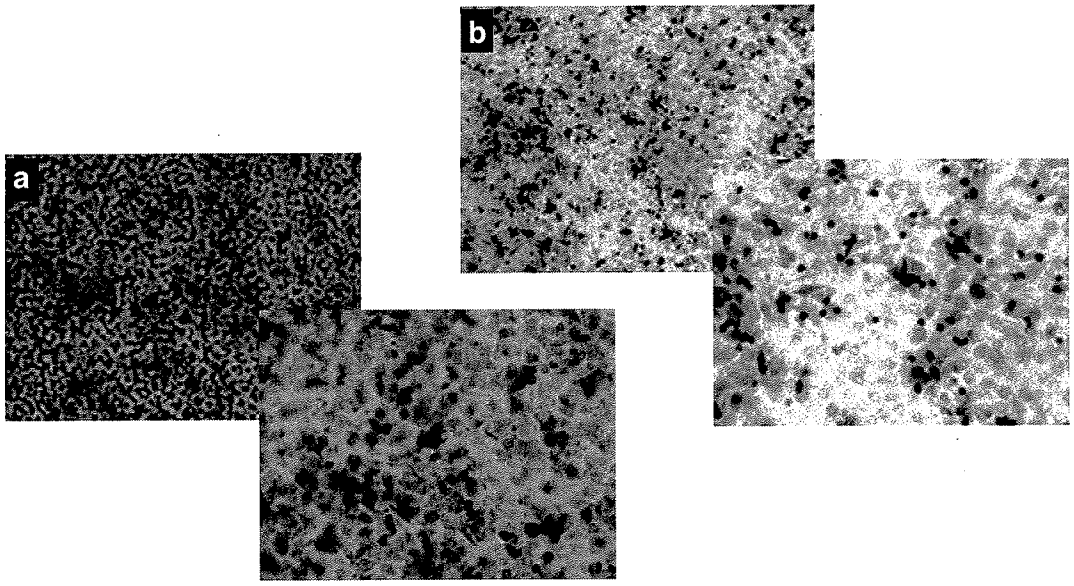

Figure 3 Microscopic appearance of CRFK/tas/pLTR1/(FFV) cell clones following β-gal staining. (a) Cell line with 50% blue cells, (b) Cell line with 30% blue cells. (Magnification ×100 and ×200).

and coevolution with their hosts (Herchenroder et al., 1994; Schweizer and Neumann-Haefelin, 1995) and this may apply to FFV and cats. Experimental infections in cats have provided no evidence of pathology or clinical signs of disease (Alke et al., 2000; Schwantes et al., 2002). Thus, although the issue of pathogenicity cannot be completely ruled out, most evidence suggests the virus is non-pathogenic. We are currently investigating the possibility of pathogenicity associated with FFV experimental infection of cats.

The paper describes the initial phases of the production of a *tas*-deleted, replication incompetent vector system, reliant on a Tas packaging cell line. Work to date has produced a functional, homogenous CRFK/tas helper cell line and current work is assessing the function of eGFP containing vectors. Preliminary results have indicated a failure of passage of the vectors, despite adequate transfection efficiencies. This could be due to a packaging problem, the 470 bp deleted *tas* fragment having been replaced with a 1.4 kb eGFP cassette. Alternatively, the disruption of Bet (a splice protein produced at high levels during viral replication, see Fig. 1), due to the *tas* deletion, may be responsible. Bet has been shown to be indispensable for in vitro FFV replication (Alke et al., 2001). Current studies are looking at supplying Bet *in trans* and reducing the foreign gene insert size to investigate these issues.

There have been various phases in FV vector development by other workers to date. Initial work looked at *tas*-deleted systems, where the accessory genes±portions of *env* were replaced with a reporter gene or gene of interest, then *tas* supplied *in trans* (Bieniasz et al., 1997; Russell and Miller, 1996). Replication competent vectors were produced by substituting the *orf2* gene with the gene of interest, retaining *tas* (Schmidt and Rethwilm, 1995). The inserted gene was expressed as a fusion protein to Bet, with either a coding sequence from the self-cleaving FMD virus 2A protease or an internal ribosomal entry site to allow recombinant protein production. These foreign genes were placed under the control of a heterologous promoter to enable high level expression.

More recently, vectors have been developed by replacing the U3 region of the 5′ LTR with the CMV promoter, allowing Tas-independent vector production at high levels (Trobridge and Russell, 1998). On reverse transcription, the 5′ U3 is restored, producing double stranded vector DNA that integrates, but as the virus is *tas*-deleted, there is no viral replication. Additional deletion of Tas responsive elements and the TATA box from the 3′ U3 region eliminates the risk of activation of the FV U3 promoter by cellular components (Heinkelein et al., 2002). Finally, minimal construct vectors have been produced using PFV, which depend on a co-transfection system to supply *gag*, *pol* and *env in trans* (Trobridge et al., 2002). FFV appears to be more difficult to manipulate than PFV in vector development, there being striking biological differences between the two viruses, particularly in the

absolute requirement of Bet for FFV replication (Alke et al., 2001). However, FFV vectors are being developed, and have been applied recently in an experimental study looking at vaccination against feline calicivirus (Schwantes et al., 2003).

Application of these vectors to tackling the problems of vaccine development for FIP is a new approach, aimed at potentiating the cell mediated immune response and provide persistent protection in the face of high environmental FCoV load. Further development of the vector system is required. Identification and cloning of coronavirus epitopes which are neutralising, but not enhancing, using FCoV serotype I strains, will enable characterisation of FCoV antigens which are most likely to give an effective vaccine response. Construction and assessment of FFV/FCoV antigen vectors will allow the determination of the value of these constructs as potential vaccines.

Thus, FFV has shown its potential for development as a viral vector and vaccination against FIP using such a vector is an exciting application of gene therapy technology in the cat. Since this is a new area of biotechnology in feline medicine, further investigation into issues of biosafety and applications need to be investigated before this technology becomes available to the clinician.

References

Alke, A., Schwantes, A., Kido, K., Flotenmeyer, M., Flugel, R.M., Lochelt, M., 2001. The Bet gene of feline foamy virus is required for virus replication. Virology 287, 310–320.

Alke, A., Schwantes, A., Zemba, M., Flugel, R.M., Lochelt, M., 2000. Characterization of the humoral immune response and virus replication in cats experimentally infected with feline foamy virus. Virology 275, 170–176.

Bandecchi, P., Matteucci, D., Baldinotti, F., Guidi, G., Abramo, F., Tozzini, F., Bendinelli, M., 1992. Prevalence of feline immunodeficiency virus and other retroviral infections in sick cats in Italy. Veterinary Immunology and Immunopathology 31, 337–345.

Barlough, J.E., Johnson-Lussenburg, C.M., Stoddart, C.A., Jacobson, R.H., Scott, F.W., 1985. Experimental inoculation of cats with human coronavirus 229E and subsequent challenge with feline infectious peritonitis virus. Canadian Journal of Comparative Medicine 49, 303–307.

Barlough, J.E., Stoddart, C.A., Sorresso, G.P., Jacobson, R.H., Scott, F.W., 1984. Experimental inoculation of cats with canine coronavirus and subsequent challenge with feline infectious peritonitis virus. Laboratory Animal Science 34, 592–597.

Bieniasz, P.D., Erlwein, O., Aguzzi, A., Rethwilm, A., McClure, M.O., 1997. Gene transfer using replication-defective human foamy virus vectors. Virology 235, 65–72.

Christianson, K.K., Ingersoll, J.D., Landon, R.M., Pfeiffer, N.E., Gerber, J.D., 1989. Characterisation of a temperature sensitive feline infectious peritonitis coronavirus. Archives of Virology 109, 185–196.

Fiscus, S.A., Teramoto, Y.A., 1987. Antigenic comparison of feline coronavirus isolates: evidence for markedly different peplomer glycoproteins. Journal of Virology 61, 2607–2613.

Flower, R.L.P., Wilcox, G.E., Cook, R.D., Ellis, T.M., 1985. Detection and prevalence of serotypes of feline syncytial spumaviruses. Archives of Virology 83, 53–63.

Glansbeek, H.L., Haagmans, B.L., Lintelo, E.G.T., Egberink, H.F., Duquesne, V., Aubert, A., Horzinek, M.C., Rottier, P.J.M., 2002. Adverse effects of feline IL-12 during DNA vaccination against feline infectious peritonitis virus. Journal of General Virology 83, 1–10.

Helps, C.R., Harbour, D.A., 1997. Comparison of the complete sequence of feline spumavirus with those of the primate spumaviruses reveals a shorter gag gene. Journal of General Virology 78, 2549–2564.

Heneine, W., Schweizer, M., Sandstrom, P., Folks, T.M., 2003. Human infection with foamy viruses, in: Rethwilm, A. (Ed.), Foamy Viruses. Berlin and Heidelberg. Springer-Verlag, pp. 181–196.

Heneine, W., Switzer, W.M., Sandstrom, P., Brown, J., Vedapuri, S., Schable, C.A., Khan, A.S., Lerche, N.W., Schweizer, M., Neumann-Haefelin, D., Chapman, L.E., Folks, T.M., 1998. Identification of a human population infected with simian foamy viruses. Nature Medicine 4, 403–407.

Heinkelein, M., Dressler, M., Jarmy, G., Rammling, M., Imrich, H., Thurow, J., Lindemann, D., Rethwilm, A., 2002. Improved primate foamy virus vectors and packaging constructs. Journal of Virology 76, 3774–3783.

Herchenroder, O., Renne, R., Loncar, D., Cobb, E.K., Murthy, K.K., Schneider, J., Mergia, A., Luciw, P.A., 1994. Isolation, cloning, and sequencing of simian foamy viruses from chimpanzees (Sfvcpz)—high homology to human foamy virus (Hrv). Virology 201, 187–199.

Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., Koyama, H., 1992. The prevalence of types I and II feline coronavirus infections in cats. Journal of Veterinary Medical Science 54, 557–562.

Hohdatsu, T., Okada, S., Koyama, H., 1991. Characterization of monoclonal antibodies against feline infectious peritonitis virus type II and antigenic relationship between feline, porcine, and canine coronaviruses. Archives of Virology 117, 85–95.

Lagaye, S., Vexiau, P., Morozov, V., Guenebautclaudet, V., Tobalytapiero, J., Canivet, M., Cathelineau, G., Peries, J., Emanoilravier, R., 1992. Human spumaretrovirus-related sequences in the DNA of leukocytes from patients with Graves-disease. Proceedings of the National Academy of Sciences of the United States of America 89, 10070–10074.

McArdle, F., Tennant, B., Bennett, M., Kelly, D.F., Gaskell, C.J., Gaskell, R.M., 1995. Independent evaluation of a modified live FIPV vaccine under experimental conditions (University of Liverpool experience). Feline Practice 23, 67–71.

Olsen, C.W., 1993. A review of feline infectious peritonitis virus: molecular biology, immunopathogenesis, clinical aspects, and vaccination. Veterinary Microbiology 36, 1–37.

Pedersen, N.C., Black, J.W., 1983. Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. American Journal of Veterinary Research 44, 229–234.

Pedersen, N.C., Pool, R.R., O'Brien, T., 1980. Feline chronic progressive polyarthritis. American Journal of Veterinary Research 41, 522–535.

Pyykkö, I., Vesanen, M., Asikainen, K., Koskiniemi, M., Airaksinen, L., Vaheri, A., 1993. Human spumaretrovirus in the etiology of sudden hearing-loss. Acta Oto-Laryngologica 113, 109–112.

Russell, D.W., Miller, A.D., 1996. Foamy virus vectors. Journal of Virology 70, 217–222.

Schmidt, M., Rethwilm, A., 1995. Replicating foamy virus-based vectors directing high-level expression of foreign genes. Virology 210, 167–178.

Schwantes, A., Ortlepp, I., Lochelt, M., 2002. Construction and functional characterization of feline foamy virus-based retroviral vectors. Virology 301, 53–63.

Schwantes, A., Truyen, U., Weikel, J., Weiss, C., Lochelt, M., 2003. Application of chimeric feline foamy virus-based retroviral vectors for the induction of antiviral immunity in cats. Journal of Virology 77, 7830–7842.

Schweizer, M., Neumann-Haefelin, D., 1995. Phylogenetic analysis of primate foamy viruses by comparison of pol sequences. Virology 207, 577–582.

Scott, F.W., Olsen, C.W., Corapi, W.V., 1995. Antibody-dependent enhancement of feline infectious peritonitis virus-infection. Feline Practice 23, 77–80.

Stoddart, C.A., Barlough, J.E., Baldwin, C.A., Scott, F.W., 1988. Attempted immunisation of cats against feline infectious peritonitis using canine coronavirus. Research in Veterinary Science 45, 383–388.

Trobridge, G.D., Russell, D.W., 1998. Helper-free foamy virus vectors. Human Gene Therapy 9, 2517–2525.

Trobridge, G., Josephson, N., Vassilopoulos, G., Mac, J., Russell, D.W., 2002. Improved foamy virus vectors with minimal viral sequences. Molecular Therapy 6, 321–328.

Vennema, H., de Groot, R.J., Harbour, D.A., Dalderup, M., Gruffydd-Jones, T., Horzinek, M.C., Spaan, W.J., 1990. Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. Journal of Virology 64, 1407–1409.

Werner, J., Gelderblom, H., 1979. Isolation of foamy virus from patients with de Quervain thyroiditis. Lancet 2, 258–259.

Winkler, I., Bodem, J., Haas, L., Zemba, M., Delius, H., Flower, R., Flugel, R.M., Lochelt, M., 1997. Characterization of the genome of feline foamy virus and its proteins shows distinct features different from those of primate spumaviruses. Journal of Virology 71, 6727–6741.

Winkler, I.G., Flugel, R.M., Lochelt, M., Flower, R.L., 1998. Detection and molecular characterisation of feline foamy virus serotypes in naturally infected cats. Virology 247, 144–151.

Winkler, I.G., Lochelt, M., Flower, R.L., 1999. Epidemiology of feline foamy virus and feline immunodeficiency virus infections in domestic and feral cats: a seroepidemiological study. Journal of Clinical Microbiology 37, 2848–2851.

Zenger, E., Brown, W.C., Song, W., Wolf, A.M., Pedersen, N.C., Longnecker, M., Li, J., Collisson, E.W., 1993. Evaluation of cofactor effect of feline syncytium-forming virus on feline immunodeficiency virus infection. American Journal of Veterinary Research 54, 713–718.